526 Rec'd PCT/PTO 05 JUL 2001

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PATENT & TRADEMARK OFFICE

(1390 REV. 5-93) US DEPT. OF COMMERCE PATENT & TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER
110024

U.S. APPLICATION NO.
(if known, sec 37 C.F.R.1.5)
09/869796

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/FR00/00011	January 5, 2000	January 5, 1999

TITLE OF INVENTION
FUNCTIONALISED POLYNUCLEOTIDE COMPOUND, OPTIONALLY MARKED AND METHOD FOR DETECTING A TARGET NUCLEIC ACID

APPLICANT(S) FOR DO/EO/US
Eric DEFRANC, Ali LAAYOUN, Jean LHOMME, Emmanuelle TREVISIOL

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 b. ☒ has been transmitted by the International Bureau.
 c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)

6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 b. ☐ have been transmitted by the International Bureau.
 c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 d. ☐ have not been made and will not be made.

8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. ☐ A FIRST preliminary amendment.

 ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☐ A substitute specification.

15. ☐ Entitlement to small entity status is hereby asserted.

16. ☐ Other items or information:

09/869796 , 091901

JC18 Rec'd PCT/PTO 05 JUL 2001

U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.5) 09/869796	INTERNATIONAL APPLICATION NO. PCT/FR00/00011	ATTORNEY'S DOCKET NUMBER 110024

17. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National fee (37 CFR 1.492(a)(1)-(5)):		
Search Report has been prepared by the EPO or JPO$860.00		
International preliminary examination fee paid to USPTO (37 CFR1.482)$690.00		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))$710.00		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....$1,000.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4).....$ 100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$860.00	
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

Claims	Number Filed	Number Extra	Rate		
Total Claims	27 - 20 =	7	X $ 18.00	$126.00	
Independent Claims	2 - 3 =	0	X $ 80.00	$	
Multiple dependent claim(s)(if applicable)			+ $270.00	$	
TOTAL OF ABOVE CALCULATIONS =				$986.00	
Reduction by 1/2 for filing by small entity, if applicable.			-	$	
SUBTOTAL =				$986.00	
Processing fee of $130.00 for furnishing the English translation later than ☐ 20 ☐ 30 month from the earliest claimed priority date (37 CFR 1.492(f)).			+	$	
TOTAL NATIONAL FEE =				$986.00	
				Amount to be refunded	$
				Charged	$

a. ☒ Check No. 120631 in the amount of $986.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Director is hereby authorized to charge any additional fees which may be required, or credit any overpayment, to Deposit Account No. 15-0461. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
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P.O. Box 19928
Alexandria, Virginia 22320

NAME: William P. Berridge
REGISTRATION NUMBER: 30,024

NAME: Joel S. Armstrong
REGISTRATION NUMBER: 36,430

Date: July 5, 2001

(1390 Rev.10-00)

PTO/PCT Rec'd 19 SEP 2001
09/869796

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of

Eric DEFRANCQ et al.

Application No.: 09/869,796

Filed: September 19, 2001 Docket No.: 110024

For: FUNCTIONALISED POLYNUCLEOTIDE COMPOUND, OPTIONALLY MARKED AND METHOD FOR DETECTING A TARGET NUCLEIC ACID

PRELIMINARY AMENDMENT

Director of the U.S. Patent and Trademark Office
Washington, D. C. 20231

Sir:

Prior to initial examination, please amend the above-identified application as follows:

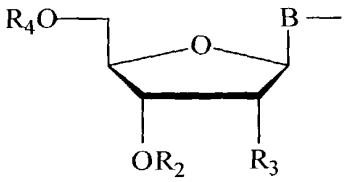
IN THE CLAIMS:

Please replace claims 4-6, 8-10, 16-18, 20-22, 24 and 26-27 as follows:

4/ (Amended) Compound according to claim 1, characterized in that L comprises at least eight atoms.

5/ (Amended) Compound according to claim 1, characterized in that L is a saturated or unsaturated hydrocarbon-based chain, optionally interrupted by at least one function chosen from amine, amide and oxy functions.

6/ (Amended) Compound according to claim 1, characterized in that W corresponds to the general formula (II)



(II)

in which:

- R_2 represents H or a protective group,

- R_3 represents H, F, OH, SH, NH_2, OCH_3 or OR_5 in which R_5 represents a protective group or an alkyl chain,

- R_4 represents an H radical, a protective group or a mono-, di- or triphosphate group,

- W being attached to L via B.

8/ (Amended) Compound according to claim 6, characterized in that R_2 is an H, R_3 is an OH group and R_4 is a triphosphate group.

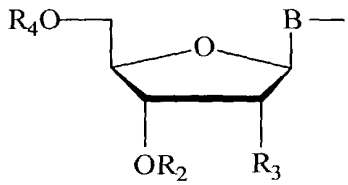
9/ (Amended) Compound according to claim 6, characterized in that R_2 is a 2-cyanoethyl-N,N-diisopropylphosphoramidite group and R_3 is H or OR_5 in which R_5 is a protective group used in oligoribonucleotide synthesis and R_4 is a 4,4'-dimethoxytrityl group.

10/ (Amended) Functionalized polynucleotide comprising at least one functionalized compound according to claim 1.

16/ (Amended) Polynucleotide according to claim 13, characterized in that L comprises at least eight atoms.

17/ (Amended) Polynucleotide according to claim 13, characterized in that L is a saturated or unsaturated hydrocarbon-based chain, optionally interrupted by at least one function chosen from amine, amide and oxy functions.

18/ (Amended) Polynucleotide according to claim 13, characterized in that W corresponds to the general formula (II)



(II)

in which:

- R_2 represents H or a protective group,

- R_3 represents H, F, OH, SH, NH_2, OCH_3 or OR_5 in which R_5 represents a protective group or an alkyl chain,

- R_4 represents an H radical, a protective group or a mono-, di- or triphosphate group,

- W being attached to L via B.

20/ (Amended) Polynucleotide according to claim 18, characterized in that R_2 is an H, R_3 is an OH group and R_4 is a triphosphate group.

21/ (Amended) Compound according to claim 18, characterized in that R_2 is a 2-cyanoethyl-N,N-diisopropylphosphoramidite group and R_3 is H or OR_5 in which R_5 is a protective group used in oligoribonucleotide synthesis and R_4 is a 4,4'-dimethoxytrityl group.

22/ (Amended) Polynucleotide according to claim 13, characterized in that the labeling reagent comprises a hydrazine or alkoxyamine function.

24/ (Amended) Method for detecting a target nucleic acid, characterized in that this target nucleic acid is brought into contact with at least one functionalized nucleotide as defined in claim 13, in the presence of elements and under conditions required for producing a polynucleotide, so as to produce a functionalized polynucleotide; the polynucleotide obtained is labeled with a labeling reagent; and then said labeled polynucleotide is detected.

26/ (Amended) Method for detecting a target nucleic acid, characterized in that this target nucleic acid is brought into contact with a functionalized polynucleotide according to claim 10; the labeling reagent is reacted; and the presence of the target nucleic acid is detected.

27/ (Amended) Method for detecting a target nucleic acid, characterized in that a labeled polynucleotide according to claim 13 is available for use, this target nucleic acid is brought into contact with the labeled polynucleotide; and the presence of the target nucleic acid is detected.

REMARKS

Claims 1-27 are pending. Claims 4-6, 8-10, 16-18, 20-22, 24 and 26-27 are amended to eliminate multiple dependencies. Prompt and favorable consideration on the merits is respectfully requested.

The attached Appendix includes marked-up copies of each rewritten claim (37 C.F.R. §1.121(c)(1)(ii)).

Respectfully submitted,

William P. Berridge
Registration No. 30,024

Thomas J. Pardini
Registration No. 30,411

WPB:TJP/zmc

Attached: APPENDIX

Date: September 19, 2001

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DEPOSIT ACCOUNT USE AUTHORIZATION
Please grant any extension necessary for entry;
Charge any fee due to our Deposit Account No. 15-0461

APPENDIX

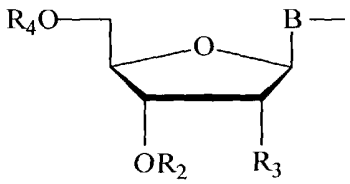
Changes to Claims:

The following are marked-up versions of the amended claims:

4/ (Amended) Compound according to claim 1~~any one of claims 1 to 3~~, characterized in that L comprises at least eight atoms.

5/ (Amended) Compound according to claim 1~~any one of claims 1 to 4~~, characterized in that L is a saturated or unsaturated hydrocarbon-based chain, optionally interrupted by at least one function chosen from amine, amide and oxy functions.

6/ (Amended) Compound according to claim 1~~any one of claims 1 to 5~~, characterized in that W corresponds to the general formula (II)



(II)

in which:

- R_2 represents H or a protective group,
- R_3 represents H, F, OH, SH, NH_2, OCH_3 or OR_5 in which R_5 represents a protective group or an alkyl chain,
- R_4 represents an H radical, a protective group or a mono-, di- or triphosphate group,
- W being attached to L via B.

8/ (Amended) Compound according to claim 6~~claims 6 and 7~~, characterized in that R_2 is an H, R_3 is an OH group and R_4 is a triphosphate group.

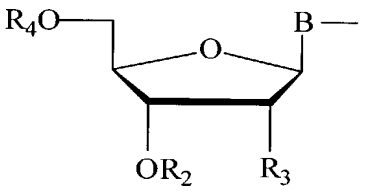
9/ (Amended) Compound according to claim 6~~claims 6 and 7~~, characterized in that R_2 is a 2-cyanoethyl-N,N-diisopropylphosphoramidite group and R_3 is H or OR_5 in which R_5 is a protective group used in oligoribonucleotide synthesis and R_4 is a 4,4'-dimethoxytrityl group.

10/ (Amended) Functionalized polynucleotide comprising at least one functionalized compound according to claim 1~~any one of the preceding claims~~.

16/ (Amended) Polynucleotide according to claim 13~~any one of claims 13 to 15~~, characterized in that L comprises at least eight atoms.

17/ (Amended) Polynucleotide according to claim 13~~any one of claims 13 to 16~~, characterized in that L is a saturated or unsaturated hydrocarbon-based chain, optionally interrupted by at least one function chosen from amine, amide and oxy functions.

18/ (Amended) Polynucleotide according to claim 13~~any one of claims 13 to 17~~, characterized in that W corresponds to the general formula (II)



(II)

in which:

- R_2 represents H or a protective group,
- R_3 represents H, F, OH, SH, NH_2, OCH_3 or OR_5 in which R_5 represents a protective group or an alkyl chain,
- R_4 represents an H radical, a protective group or a mono-, di- or triphosphate group,
- W being attached to L via B.

20/ (Amended) Polynucleotide according to claim 18~~claims 18 and 19~~, characterized in that R_2 is an H, R_3 is an OH group and R_4 is a triphosphate group.

21/ (Amended) Compound according to claim 18~~ and 19~~, characterized in that R_2 is a 2-cyanoethyl-N,N-diisopropylphosphoramidite group and R_3 is H or OR_5 in which R_5 is a protective group used in oligoribonucleotide synthesis and R_4 is a 4,4'-dimethoxytrityl group.

22/ (Amended) Polynucleotide according to claim 13~~any one of claims 13 to 21~~, characterized in that the labeling reagent comprises a hydrazine or alkoxyamine function.

24/ (Amended) Method for detecting a target nucleic acid, characterized in that this target nucleic acid is brought into contact with at least one functionalized nucleotide as defined in claim 13~~any one of claims 13 to 21~~, in the presence of elements and under conditions required for producing a polynucleotide, so as to produce a functionalized

polynucleotide; the polynucleotide obtained is labeled with a labeling reagent; and then said labeled polynucleotide is detected.

26/ (Amended) Method for detecting a target nucleic acid, characterized in that this target nucleic acid is brought into contact with a functionalized polynucleotide according to claim 10~~any one of claims 10 to 12~~; the labeling reagent is reacted; and the presence of the target nucleic acid is detected.

27/ (Amended) Method for detecting a target nucleic acid, characterized in that a labeled polynucleotide according to claim 13~~any one of claims 13 to 23~~ is available for use, this target nucleic acid is brought into contact with the labeled polynucleotide; and the presence of the target nucleic acid is detected.

FUNCTIONALIZED COMPOUND, OPTIONALLY LABELED POLYNUCLEOTIDE AND METHOD FOR DETECTING A TARGET NUCLEIC ACID

The present invention relates to a novel nucleoside or nucleotide compound functionalized with an alkyl ketone group, to a polynucleotide comprising at least one nucleotide unit functionalized with an alkyl ketone group, before and after labeling, and also to the implementation and the uses of these products in particular for detecting nucleic acid sequences.

In the nucleic acids field, the synthesis of functionalized nucleotides has been described in particular in the diagnostic field and more particularly for preparing labeled nucleic acid probes which may be used for detecting a target nucleic acid.

Two main types of problem arise in producing these probes. Initially, the functionalized nucleotide must be incorporated into a polynucleotide. Next, the function carried by the nucleotide must be able to react with a tracer in a way which is specific and effective for using the polynucleotide as a detection probe.

Thus, EP-A-0 407 816 describes uracil derivatives modified at position 5 for producing probes via the chemical or enzymatic route. Still with the same aim, patents WO-A-86/06726 and EP-A-0 212 951 describe cytosine derivatives modified at position 4. Patent EP-A-0 254 646 describes an adenosine derivative modified at position 8.

Patent WO-A-92/00989 describes a specific use of modified nucleotides for introducing proteins onto a polynucleotide.

Patent application WO-A-98/05766 by the applicant poses the problem of the incorporation of functionalized nucleotides which may be incorporated using enzymatic

reaction and in particular using enzymatic amplification techniques, no longer for preparing labeled probes, but directly for generating a labeled target. In this case, there is a greater need for sensitivity and the choice of the functionalized nucleotide is essential for producing the appropriate sensitivity.

A certain number of nucleophilic functions, such as amine and alkoxyamine functions, or electrophilic functions, such as the aldehyde function, are described which allow effective incorporation of the functionalized nucleotide in the course of amplification, but there nevertheless remains a need for a functionalized nucleotide which is even more effective, in particular in terms of ease of preparation, in terms of neutrality with respect to the enzymatic or chemical reactions and in terms of reactivity for the labeling of this nucleotide with a tracer after incorporation.

It has been found, by the applicant, that, surprisingly, the alkyl ketone function answers the abovementioned drawbacks.

In fact, while the prior art describes the use of R-CO- units in which R is an alkyl group, in the case of oligonucleotide synthesis (WO-A-93/22326), the aim of this function is to protect the exocyclic amine of the bases. At the end of synthesis, the amine is deprotected by the action of an alkali agent.

By way of illustration of this state of the art, the following documents may be mentioned. The article by K.K. Ogilvie and M.J. Nemer, Tetrahedron Letters, vol. 21, (1980) pages 4145-4148, discloses, as an intermediate of synthesis of a nucleotide, a nucleotide compound carrying a levulinoyl group attached to the hydroxyl function on

the 3' side of the pentose. The aim of introducing this group is to protect the OH function, and it is then immediately removed after said compound has been obtained. The article by C.T.J. Wreesmann et al., Nucleic Acids Research, vol. 11, N°23, (1983) pages 8389-8405, describes the production of a synthetic dinucleotide intermediate, the hydroxyl functions of which are protected by the levulinoyl group. In the presence of aqueous ammonia, the hydroxyl functions are released.

The alkyl ketone function as defined in the present invention is sufficiently stable to withstand this type of treatment and does not therefore represent a protective group, and its stability with respect to the various methods of chemical synthesis results in greater ease of preparation, in particular with respect to amine, aldehyde or alkoxyamine functions, as described in patent WO-A-98/05766.

Similarly, in the abovementioned patents (EP-A-0 407 816, WO-A-86/06726, EP-A-0 212 951, EP-A-0 254 646 and WO-A-92/00989), the functions described for functionalizing the nucleotides are chosen from functions which are reactive from a chemical point of view, such as amine and thiol functions, which are optionally protected. If these functions are protected, a deprotection step is necessary, which complicates the labeling step. If these functions are free, inhibition of the enzymatic reaction or of the side reactions in the case of chemical synthesis may occur.

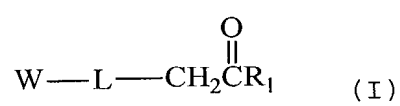
This chemical stability of the alkyl ketone function should not make it a good candidate for a reaction for coupling to a label, and yet the present invention demonstrates that, surprisingly, this function

is reactive with regard to labeling and that, in addition, the detection of the product after labeling is very sensitive.

Finally, the nucleotides carrying this function exhibit excellent neutrality with respect to enzymatic reactions since it is possible to completely replace a natural nucleotide with a nucleotide carrying this alkyl ketone function in an enzymatic reaction, without affecting the yield of this reaction and, even more surprisingly, in certain cases improving it.

Document WO-A-95/24185 describes a nucleoside modified with an alkyl ketone group, the alkyl component of which may comprise up to 20 carbon atoms. This compound is in particular intended for the synthesis of oligonucleotides which find an application in therapy, in diagnosis. The introduction of a group, in particular an alkyl ketone group, onto the pyrimidine nucleus of the nucleoside described is not directed toward functionalization in view of a subsequent reaction of said group, but is aimed at producing oligonucleotide analogs which, with respect to the natural oligonucleotides, exhibit properties which are advantageous in terms of their uses, such as greater ease of hybridization with a target nucleic acid or greater resistance to nucleases.

The object of the present invention is to describe a novel functionalized compound which comprises an alkyl ketone function and which has the formula (I) below:



(I)

in which

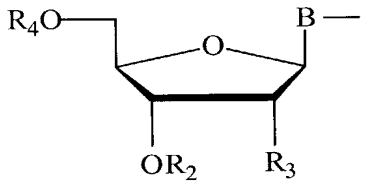
- W represents a nucleotide analog;

- L represents a linker arm between W and the alkyl ketone group, comprising at least four atoms, advantageously at least eight atoms; L is in particular chosen from saturated or unsaturated hydrocarbon-based chains, optionally interrupted by at least one amine, amide or oxy function; preferably, the linker arm comprises a chain of 8 to 30 atoms; in particular, the linker arm comprises from 8 to 20 atoms, at least one of which is an amide function;

- R_1 represents a linear or branched alkyl chain, preferably an alkyl chain having at most 6 carbons; advantageously, R_1 is a methyl group.

The term "nucleotide analog" is intended to mean a nucleoside or a nucleotide, a nucleoside or a nucleotide carrying one or more modifications on one of the constituent elements of said nucleoside or nucleotide, for instance a modification of the deoxyribose or ribose sugar, in particular xylose, arabinose, sugars with an alpha configuration (FR 2 607 507), PNAs (M. Egholm et al., J. Am. Chem. Soc., (1992), **114**, 1895-1897), sugar analogs such as 4'-thio-ribose or -deoxyribose, or sugars with a D or L configuration; a modification of the nitrogen-containing base; a modification of the phosphate or of its equivalent for the nucleotides and also all of the protective groups used in the chemical synthesis.

Advantageously, the compound corresponds to the formula (I) in which W has the general formula (II):



(II)

in which:

- R_2 represents H or a protective group;
- R_3 represents H, F, OH, SH, NH_2, OCH_3 or OR_5 in which R_5 represents a protective group or an alkyl chain;
- R_4 represents an H radical, a protective group or a mono-, di- or triphosphate group;
- B represents a nitrogen-containing base,
- W being attached to L via B.

The nitrogen-containing base is chosen in particular from purines or pyrimidines, such as adenine, guanine, uracil, cytosine or thymine, or any other modified base allowing hybridization, for instance natural modified bases (such as 6-keto purine, xanthine, 5-methylcytosine or 2-aminopurine) or unnatural modified bases (such as thioguanine or 8-oxoguanine, deazapurine or azapurine), or analogs of bases such as universal bases (such as nebularin, nitroindole or nitropyrrole derivatives). Certain functions of the bases likely to interfere with the strategies of solid- or liquid-phase chemical synthesis may be protected with suitable protective groups.

Preferably, the nitrogen-containing base is adenine, uracil or cytosine.

The linker arm L is grafted onto any position of the nitrogen-containing base or of its analog. Preferably, the linker arm will be grafted onto a position which does

not disturb hybridization. In particular, the linker arm will be attached to the amine at position 4 of cytosine, position 5 of uracil or the amine at position 6 of adenine.

The term "protective group" is intended to mean the groups conventionally used in the chemical synthesis of nucleosides, nucleotides and oligonucleotides (see, for example, Chemistry of Nucleosides and Nucleotides, Edited by Leroy B. Townsend, Plenum Press, New York and London and Protocols for Oligonucleotides and Analogs, Synthesis and Properties, Edited by S. Agrawal, Humana Press, Totowa, New Jersey). Preferably, in the case of chemical synthesis, R_4 is a 4,4'-dimethoxytrityl group and R_2 is a 2-cyanoethyl-N,N-diisopropylphosphoramidite group and R_3 is H or OR_5, in which R_5 is a protective group used in oligoribonucleotide synthesis.

Preferably, in the case of enzymatic synthesis, R_4 is a triphosphate group, R_2 is H and R_3 is OH.

The phosphate groups are generally in the form of salts, and particularly lithium, sodium or triethylammonium acetate salts.

The invention also relates to a functionalized polynucleotide comprising at least one functionalized nucleotide as defined above. It may be synthesized by chemical and/or enzymatic reaction. In the case of synthesis by enzymatic reaction, and in particular in the case of enzymatic amplification, the neutrality of the functionalized nucleotide with respect to enzymatic reactions allows the incorporation of several functionalized nucleotides.

The term "enzymatic reaction" includes all reactions in which at least one enzyme with activity which

is related to a nucleotide is involved. It is thus intended to mean all reactions comprising at least one enzymatic step in which a nucleotide is used as the substrate for the enzyme, regardless of whether said nucleotide is transformed during this enzymatic step. By way of example, such reactions are chosen from those used in molecular biology techniques such as transcription, ligation, elongation and cleavage and more particularly in amplification techniques (see, for example, the article by E. Winn-Deen, Journal of Clinical assay, vol 19, p21-26, (1996)).

Thus, the enzymes which have activities which are related to nucleotides may, in particular, be selected from the following nonexhaustive list: DNA-dependent DNA polymerases, such as the DNA polymerase I Klenow fragment of *E. Coli*, TAQ polymerase, the T7, T4 or T5 DNA polymerases, eukaryotic cellular polymerases or viral polymerases; RNA-dependent DNA polymerases, such as AMV (avian myoblastosis virus) or MMLV (Moloney murine leukemia virus) polymerases; RNA polymerases, such as the T7, T3 SP6, N4, or PBSII RNA polymerases, or *E. Coli* RNA polymerase; enzymes with nuclease activity, such as restriction endonucleases or Rnase H; or polyA polymerases, replicases such as Q-beta-replicase, terminal transferases or ligases.

Heat-stable enzymes which have the enzymatic activities described above may also be used in the invention.

According to a preferred embodiment, techniques using a transcription step, such as NASBA (nucleic acid sequence based amplification), TMA (transcription mediated amplification) or post-PCR (polymerase chain reaction)

transcription, as described in the articles by R.J. Lipshutz et al, Biotechniques, 19(3), p442-447, 1995 or M. Kozal et al, Nature Medecine , 2(7), p753-759, 1996, will be chosen for the synthesis of the functionalized polynucleotide.

The elements and conditions required for carrying out these enzymatic reactions so as to produce a polynucleotide are well known to those skilled in the art. The manual Current Protocols in Molecular Biology, edited by F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore J.G. Seidman, J.A. Smith and K. Struhl, John Wiley & Sons, 1996 volume 1 chapter 3, gives the methods for enzymatic manipulation of DNA and RNA. Similarly, the work "Molecular Methods for Virus Detection", edited by D.L. Wiedbrand and D.H. Farkas, Academic press, San Diego, 1995 in particular in chapters 8, 9, 12, 13, 14, 15 and 16, gives examples for the enzymatic amplification techniques.

The term "chemical synthesis" is intended to mean all methods, both in solid phase and in liquid phase, in which a suitably protected nucleotide monomer reacts with another nucleotide monomer or polymer via a coupling reaction.

Chemical synthesis methods are given, for example, in "Methods in Molecular Biology, volume 20, Protocols for oligonucleotides and analogs", edited by S. Agrawal, Humana Press, Totowa, New Jersey, 1993 and "Methods in Molecular Biology, volume 26, Protocols for oligonucleotides conjugates", edited by S. Agrawal, Humana Press, Totowa, New Jersey, 1994.

The term "polynucleotide" is intended to mean a chain of at least 2 nucleotide monomers. Preferably, if

the polynucleotide is synthesized by the chemical route, it is less than 300 nucleotides, and advantageously less than 150 nucleotides, in size. Preferably, if the polynucleotide is synthesized by enzymatic reaction, it is less than 20 kb, and advantageously less than 10 kb, in size.

The two routes of synthesis, chemical synthesis and enzymatic synthesis, can be combined in order to prepare a polynucleotide.

The invention also relates to a labeled functionalized polynucleotide comprising at least one functionalized compound of general formula (I'):

$$W-[-L-]_n-CH_2COR_1 \qquad .(I')$$

in which

W represents a nucleotide analog as defined above,

L represents a linker arm comprising at least four atoms,

n represents an index equal to 0 or 1,

R_1 represents a linear or branched alkyl chain,

the alkyl ketone group of said functionalized compound having interacted with a labeling reagent.

W, L and R_1 advantageously satisfy the definitions given above to describe preferred functionalized compounds of the invention.

The term "labeling reagent" is intended to mean a tracer which directly or indirectly generates a detectable signal capable of reacting with the alkyl ketone function.

The following is a nonlimiting list of these tracers:

- enzymes which produce a signal detectable, for example, by colorimetry, fluorescence or luminescence, such as horseradish peroxidase, alkaline phosphatase, beta-galactosidase or glucose-6-phosphate dehydrogenase;

- chromophores, such as fluorescent, luminescent or dye compounds;

- groups with an electron density which can be detected by electron microscopy or through their electrical property, such as by conductivity, amperometry, voltametry, impedance measurements;

- groups which can be detected using optical methods, such as diffraction, surface plasmon resonance or contact angle variation, or physical methods, such as atomic force spectroscopy, the tunnel effect;

- radioactive molecules such as ^{32}P, ^{35}S or ^{125}I.

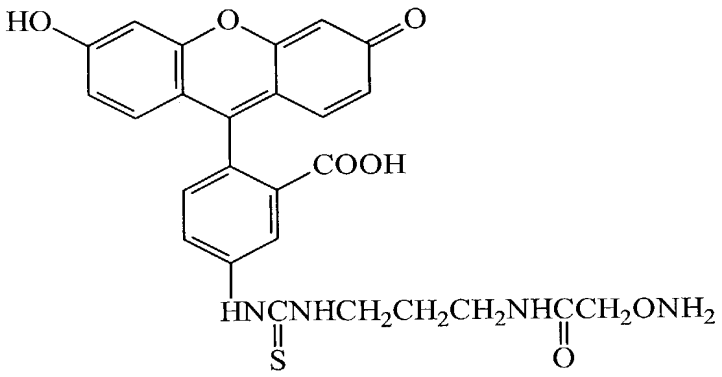
Preferably, the tracer is a fluorescent compound with low steric hindrance, such as fluorescein, dansyl, chromophores of the IR (Li-COR Inc, Lincoln NE, USA), CY5 and CY3 (Randolph J.B. and al, Nucleic Acids Res., 25(14), p2923-2929, 1997) type and derivatives thereof. The term "low steric hindrance" is intended to mean a molecular weight of less than 1000 g/mol.

In order to react with the alkyl ketone function, this labeling reagent must carry a nucleophilic function capable of reacting with an alkyl ketone function, such as alkoxyamine or hydrazine functions.

Preferably, the function chosen is alkoxyamine, which may be introduced by any direct or indirect means. The term "direct means" is intended to mean a covalent bond between the tracer, or a molecule carrying the tracer, and the alkoxyamine function. The term "indirect means" is intended to mean complexation systems of the

metal/chelate type or affinity systems, i.e. haptens which can be detected by a specific antibody or a protein, such as the biotin/avidin or streptavidin pairing or the sugar/lectin pairing. In this case, the tracer is carried by the antibody or the protein and the alkoxyamine function is carried by the hapten.

In particular, the labeling reagent has the formula:



The invention also relates to a solid support to which a nucleotide, a nucleoside or a polynucleotide according to the invention is attached by covalence.

In order to perform this attachment, a nucleotide, a nucleoside or a polynucleotide comprising an alkyl ketone group is reacted with a solid support on which there is an alkoxyamine or hydrazine function, preferably an alkoxyamine function.

In a first embodiment, the polynucleotide is preformed and the final reaction consists in grafting, at a predetermined position on the support, the polynucleotide. In one particular embodiment, the polynucleotides are synthetic oligonucleotides (synthesized via the chemical route) which are short in size (less than 50 bases), and in a second particular embodiment, the polynucleotides are more than 50 bases in

size and are prepared using enzymatic methods such as enzymatic amplification.

In a second embodiment, the nucleoside or nucleotide is added via successive steps (chain extension) on the support in order to obtain, at the end of the synthetic cycle, a polynucleotide grafted to a predetermined position on the solid support.

A preferential use of these grafted supports is the production of biochips for gene analysis.

By way of illustration, examples of these biochips are given in the publications by G. Ramsay, Nature Biotechnology, 16, p40-44, 1998; F. Ginot, Human Mutation, 10, p1-10, 1997; J. Cheng et al, Molecular diagnosis, 1(3), p183-200, 1996; T. Livache et al, Nucleic Acids Research, 22(15), p2915-2921, 1994; J. Cheng et al, Nature Biotechnology, 16, p541-546, 1998 or in patents US-A-4 981 783 (Augenlicht), US-A-5 700 637 (Southern), US-A-5 445 934 (Fodor), US-A-5 744 305 (Fodor), US-A-5 807 522 (Brown).

The invention also relates to a method for detecting a target nucleic acid in a sample, in which this target nucleic acid, optionally pretreated, is brought into contact with at least one functionalized compound corresponding to formula (I'), in the presence of the elements and under conditions required for producing a polynucleotide of the invention, so as to produce a functionalized polynucleotide; in labeling said polynucleotide with a labeling reagent and then in detecting said labeled polynucleotide. The abovementioned elements and conditions are well known to those skilled in the art.

The term "pretreatment" is intended to mean the various steps for treating the sample in order to make the target nucleic acid accessible, such as for example lyzing, fluidifying, concentrating.

Preferably, the functionalized polynucleotide is obtained via an enzymatic amplification reaction which acts on the target nucleic acid which serves as a matrix, and which is capable of incorporating the functionalized nucleotide.

Advantageously, the enzymatic amplification technique is NASBA (nucleic acid sequence based amplification), TMA (transcription mediated amplification) or post-PCR (polymerase chain reaction) transcription, as described in the articles by R.J. Lipshutz et al, Biotechniques, 19(3), p442-447, 1995 or M. Kozal et al, Nature Medecine , 2(7), p753-759, 1996.

The labeled polynucleotide may be detected qualitatively and/or quantitatively in homogeneous or heterogeneous phase. A preferential detection mode consists in attaching the labeled polynucleotide to a solid support via a hybridization reaction between the labeled polynucleotide and another polynucleotide, itself attached to the solid support, and then revealing the presence of the labeled polynucleotide after a washing step. This revelation is carried out directly by reading, such as for example with a scanner or a camera if the tracer is a fluorescent molecule.

The detection method is particularly useful when a multitude of polynucleotides are attached to the solid support at a predetermined position so as to form a "DNA chip".

Specifically, the density of the polynucleotides attached to the solid support imposes considerable steric constraints during the hybridization and the labeled polynucleotide according to the invention allows good sensitivity of detection. Examples of these chips are given, for example cited in the publications and patents mentioned above.

The detection method can be used for sequencing, profiling the expression of messenger RNAs or screening mutations, or diagnosing infectious or genetic diseases.

A fragmentation step can take place in order to promote the hybridization of the labeled polynucleotide on the DNA chip, before, together with, or after the labeling step.

The invention also relates to a method for detecting a target nucleic acid in a sample, in which this target nucleic acid is brought into contact with a functionalized polynucleotide of the invention, the labeling reagent is reacted and the presence of the target nucleic acid is detected.

Another means of carrying out the invention is to react the functionalized polynucleotide and the labeling reagent, before the hybridization with the target nucleic acid. This target nucleic acid may have been amplified using an enzymatic amplification technique.

Finally the invention relates to the method for detecting a target nucleic acid, according to which a labeled polynucleotide of the invention is available for use, the nucleic acid is brought into contact with the labeled polynucleotide and the presence of the target nucleic acid is detected.

The term "solid support" as used herein includes all materials on which it is possible to immobilize a polynucleotide for use in diagnostic tests and in separation processes. Natural or synthetic materials, which may or may not be chemically modified, may be used as a solid support, in particular polysaccharides such as cellulose-based materials, for example paper, cellulose derivatives such as cellulose acetate and nitrocellulose, dextran; polymers such as polyvinyl chlorides, polyethylenes, polystyrenes, polyacrylates, polyamides, or copolymers based on monomers of the styrene type, unsaturated carboxylic acid esters, vinylidene chloride,

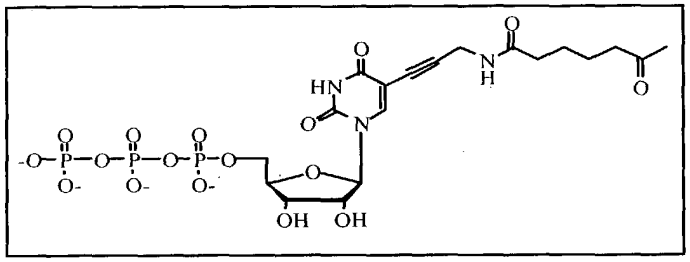
dienes or compounds with nitrile functions (such as acrylonitrile); vinyl chloride/propylene copolymers, vinyl chloride/vinyl acetate copolymers; natural fibers such as cotton and synthetic fibers such as nylon; inorganic materials such as silica, quartz, glasses, ceramics; latexes, i.e. colloidal aqueous dispersions of any water-insoluble polymer; magnetic particles; metallic derivatives, gels etc.

The following examples make it possible to illustrate some of the advantages of the invention without, however, limiting the scope thereof.

The attached figure represents the transcription yields measured as a function of the nucleotide used.

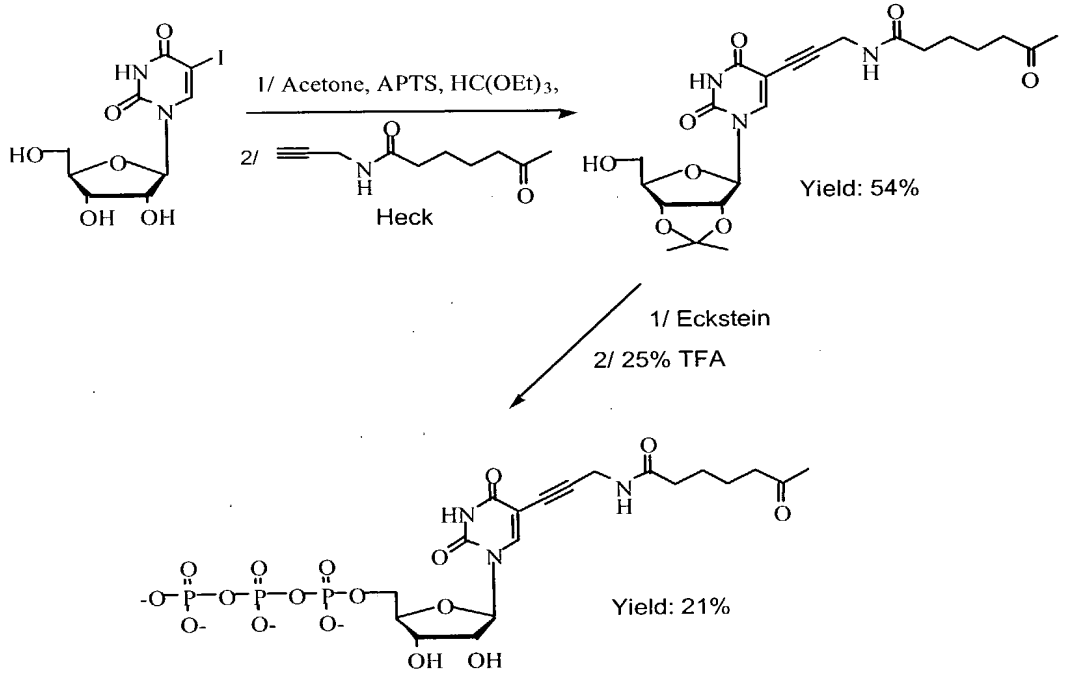
EXAMPLE I : SYNTHESIS OF METHYL KETONE NUCLEOTIDES

I.1. Synthesis of uridine(C5)-C9-methylketone 1



1

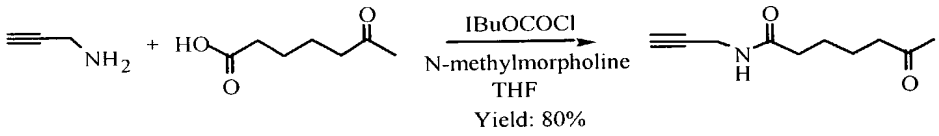
Synthetic pathway:



The 2',3'-OH positions are protected by reacting acetone in acid medium according to the protocol described in "Nucleic acid chemistry, Editors Townsend-Tipson, Wiley-Interscience, John Wiley & Sons, p 765-766 (1978)".

Synthesis of the methyl ketone chain

The methyl ketone unit is introduced onto the arm by peptide coupling between propargylamine and 6-oxo-heptanoic acid.



6-Oxoheptanoic acid (2.75 g, 18.16 mmol) is solubilized in 40 ml of anhydrous THF. The solution is placed at 0°C under an argon atmosphere. N-methyl-morpholine (2 ml, 18.16 mmol) is then added, followed by propargylamine (1.25 ml, 18.16 mmol) 15 min later, and the

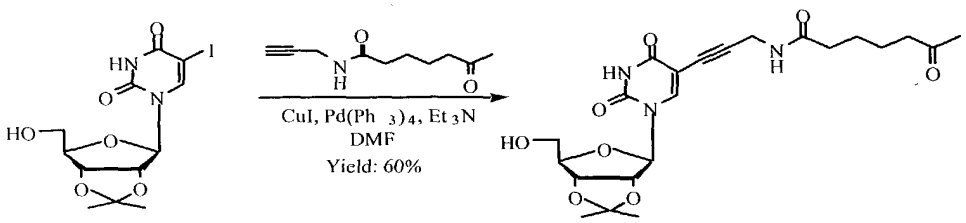
mixture is allowed to return to room temperature. After reaction for 30 minutes, the precipitate is filtered and evaporated to dryness.

The residue obtained is taken up in dichloromethane and is washed with a 0.1 N aqueous sodium hydroxide solution, then with a 1 N aqueous hydrochloric acid solution and finally with water saturated with sodium chloride.

After drying over Na_2SO_4, the dichloromethane is evaporated off and the residue is chromatographed on silica gel (eluent: ethyl acetate). The product is thus obtained in the form of a white powder (2.6 g, 14.4 mmol; 80%).

The methyl ketone chain was characterized by proton NMR, carbon 13 NMR and mass spectrometry.

Introduction of the methyl ketone chain onto the nucleoside: Heck coupling (Hobbs, *J. Org. Chem.*, 1989, 54, 3420-3422)



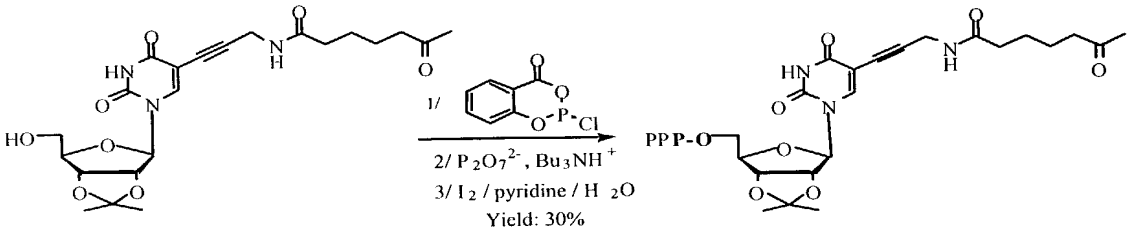
5-Iodouridine 2',3'-isopropylidene (500 mg; 1.22 mmol) and copper iodide (44 mg, 0.232 mmol) are added to 5 ml of DMF which has been degassed and placed under argon. The reaction is placed in darkness and then triethylamine (323 µl, 2.32 mmol) and the chain containing the methyl ketone function (630 mg, 3.48 mmol) are added.

The mixture is left under argon for 10 minutes. Tetrakis triphenylphosphine palladium (134 mg, 0.116 mmol) is then added. After reaction for 3 hours, the DMF is evaporated off, followed by coevaporation with acetonitrile. The residue is taken up in ethyl acetate and the organic phase is washed with an aqueous solution saturated with sodium chloride. After drying over Na_2SO_4 and evaporation, the residue is chromatographed on silica gel (eluent: ethyl acetate/methanol: 90/10).

After evaporation, the methyl ketone nucleoside is obtained in the form of a whitish powder (340 mg, 0.73 mmol, 60%). The product was characterized by proton NMR, carbon 13 NMR and mass spectrometry. The methyl ketone nucleoside correctly protected for the introduction of the triphosphate in 5' is thus obtained.

Production of the uridine(C5)-C9-methylketone triphosphate nucleoside 1

Phosphorylation: Eckstein, J. Org. Chem., ***1989,*** *54, 631-635*

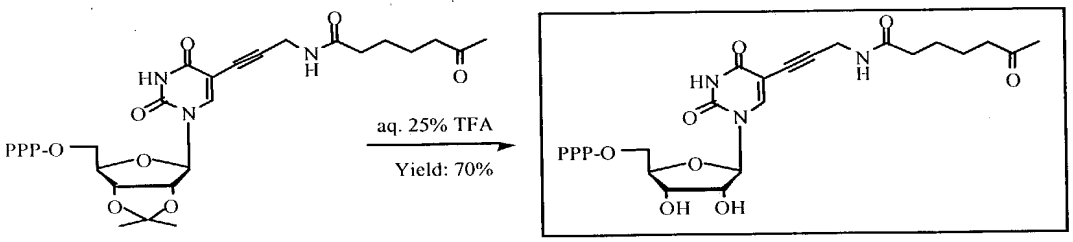


The methyl ketone nucleoside (46 mg, 0.1 mmol) is dissolved in anhydrous pyridine and evaporated twice. 100 ml of pyridine, 300 ml of dioxane and a freshly prepared solution of 2-chloro-4H-1,2,3-dioxaphosphorin-4-one (1 M) in dioxane (130 μl; 130 μmol) are then added under argon, the mixture is left to stir for 20 minutes,

and then a 0.5 M solution of tributylammonium pyrophosphate in anhydrous DMF (320 µl, 0.16 mmol) and, simultaneously, 130 µl of tributylamine are then added. After 30 minutes, 2 ml of 1% iodine solution are added to a pyridine/water (98/2: v/v) mixture.

After stirring for 20 minutes, the excess iodine is eliminated with a 5% aqueous NaHS03 solution and the stirring is continued for 10 minutes. The mixture is evaporated to dryness and an extraction is carried out with a water/dichloromethane mixture. The aqueous phase is evaporated off and then C18 reverse phase chromatography (flash) is performed (eluent: water/methanol 1/1). The fractions containing the product are evaporated and the counter-ion exchange is carried out by passing over Dowex, Na+ resin. The protected triphosphate is thus recovered (0.03 mmol; 30%).

Deprotection

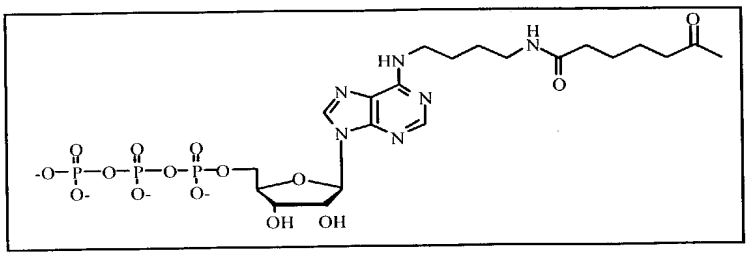


1

The protected triphosphate (0.03 mmol) is taken up in 15 ml of milli-Q water to which 15 ml of a 25% aqueous TFA solution are added. The solution is stirred for 15 minutes, then evaporated and coevaporated twice with water.

The product is taken up in 10 ml of milli-Q water and neutralized with 0.1 N sodium hydroxide, to pH 8. After the evaporation, the protected triphosphate is purified on C18 reverse phase (H_2O then H_2O/MeOH, 1/1). The counter-ion exchange is carried out by passing over cation exchange resin (Dowex Na^+). The fractions containing the product are evaporated and assayed. 0.021 mmol (70%) of the uridine(C5)-C9-methylketone triphosphate nucleoside 1 is thus recovered, and this nucleoside was characterized by proton NMR, carbon 13 NMR and phosphorus 31 NMR.

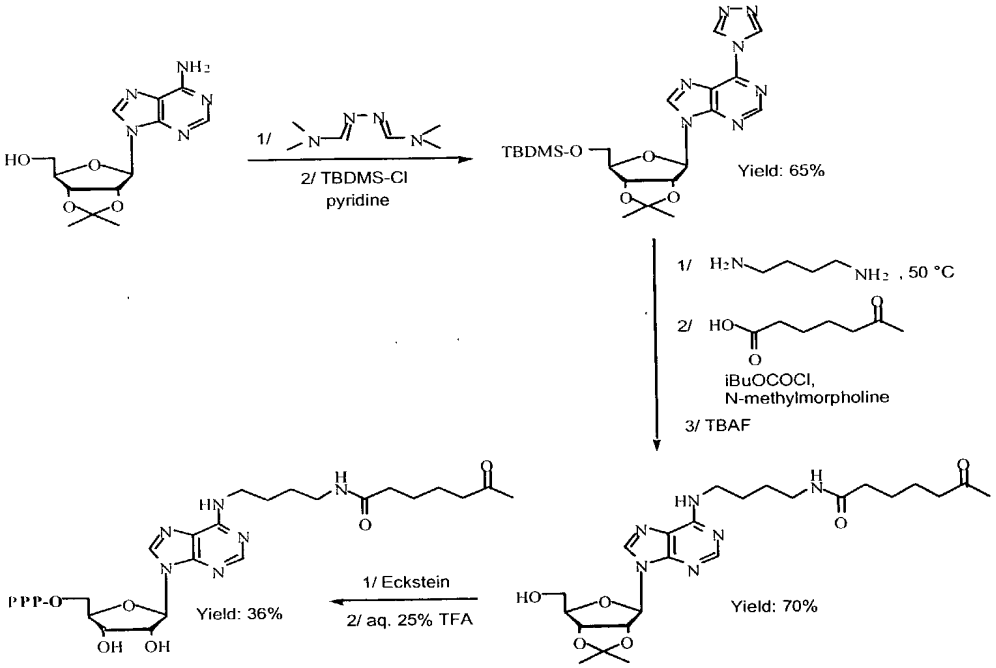
I.2. Synthesis of adenosine(N6)-C10-methylketone 2



2

Synthetic pathway

The introduction of diaminobutane at position 6 of the adenosine is carried out by substitution of the triazolo group carried by the protected intermediate nucleoside. The methyl ketone function is then introduced by peptide coupling at the nucleoside level. After deprotection of the silyl group in 5', phosphorylation is carried out by the Eckstein method. After deprotection, the expected triphosphate is obtained, which is characterized by proton NMR and phosphorus 31 NMR.



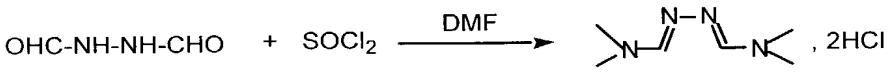
2

Synthesis of the protected triazolo nucleoside

Preparation of adenosine 2',3'-isopropylidene (Nucleic Acid Chemistry, part 2, Editors Townsend, Tipson, Wiley Interscience, John Wiley & Sons p. 768, (1978))

Ethyl orthoformate (12.44 ml, 74.8 mmol) is added dropwise, under argon, to a suspension of adenosine (5 g, 18.7 mmol) in acetone (10 ml) containing APTS (para-toluene-sulfonic acid) (3.9 g, 20.6 mmol). After reaction overnight, 110 ml of water containing 1.86 ml of aqueous ammonia at 27% are added. After stirring for 30 minutes, the reaction mixture is evaporated until white crystals appear. After 12 h at +4°C, a white precipitate is obtained which is recrystallized in water. 4.17 g (13.5 mmol, 72%) of product is obtained in the form of a white powder. This intermediate was characterized by proton NMR.

Synthesis of amidine (Bartlett and Humphreg, *J. Chem. Soc.*, 1967, 1664-1666)



Thionyl chloride (39.96 g, 24.5 ml, 0.338 mol) is added dropwise to N-N'-diformylhydrazine (12 g, 0.136 mol) in DMF (270 ml) at 10°C. The mixture becomes yellow. Stirring is maintained for 2 days. The precipitate obtained is filtered and washed with DMF then with ether. After drying under a vacuum, the amidine is obtained with a yield of 95% (28 g, 0.130 mol).

Preparation of the triazolo intermediate (Samano, Miles, Robins, *J. Am. Chem. Soc.*, 1994, *116*, 9331-9332)

The adenosine isopropylidene (1 g, 3.2 mmol) and the amidine described above (1.4 g, 6.5 mmol) are stirred into pyridine (15 ml) at 100°C under argon for 48 h. The pyridine is then evaporated off and coevaporated with toluene. The oil obtained is then taken up with ethyl acetate and this organic phase is washed with water saturated with NaCl. After drying over Na_2SO_4 and evaporation, the triazolo nucleoside is obtained in the form of a white powder with a yield of 60% (700 mg, 1.9 mmol). It was then characterized by proton NMR.

Protection in 5' of the triazolo derivative

The triazolo nucleoside (1 g, 2.8 mmol) is solubilized in 20 ml of pyridine. TBDMS - Cl (tert-butyldimethylsilyl chloride), (462 mg, 3 mmol) is added at 0°C under argon. Stirring is maintained for two hours then the pyridine is evaporated off. The residue thus obtained is chromatographed on silica gel (eluent: CH_2Cl_2/methanol : 95/5). After evaporation, the totally protected intermediate adenosine is obtained in the form of a white powder (1.25 mg, 2.6 mmol, 93%). This nucleoside was characterized by proton NMR, carbon 13 NMR and mass spectrometry.

Introduction of the diaminobutane chain onto the protected intermediate adenosine

The triazolo adenosine (1.25 mg, 2.64 mmol) is solubilized in 10 ml of acetonitrile. Diaminobutane (2.7 ml, 25.4 mmol) is then added and the mixture is stirred at 50°C under argon. After 5 hours, the solvent is evaporated off and the residue is taken up in ethyl acetate. This organic phase is washed with water saturated

with NaCl. After drying over Na_2SO_4 and evaporation by chromatography on silica gel (eluent: CH_2Cl_2/MeOH: 8/2, then CH_2Cl_2/MeOH: 8/2 in the presence of 2% aqueous ammonia).

After evaporation, the product is obtained in the form of an oil (1 g, 2 mmol, 80%).

The aminated nucleoside was characterized by proton NMR, carbon 13 NMR and mass spectrometry.

Coupling to the methyl ketone chain and deprotection in 5'

6-Oxo-heptanoic acid (288 mg, 2 mmol) is dissolved in 5 ml of anhydrous THF. The solution is placed at 0°C under argon. N-Methylmorpholine (223 µl, 2 mmol) is then added, followed by isobutyl dichloroformiate (258 µl, 2 mmol) 5 min later. After 15 minutes, the aminated nucleoside is added. After 2 hours, the THF is evaporated off and the residue is taken up with ether. This is washed with a 1 N aqueous NaOH solution and then with water saturated with NaCl. After drying over Na_2SO_4 and evaporation, an oil is obtained. The desilylation is carried out by taking the oil up in 10 ml of THF, to which TBAF (3.25 ml of a 1M solution in THF) is added. After one hour, the solvent is evaporated off. The residue is solubilized in dichloromethane and washing is carried out with water saturated with NaCl. After drying and evaporation, the residue is chromatographed on silica gel (eluent: CH_2Cl_2, then CH_2Cl_2/MeOH: 95/5).

After evaporation, 870 mg of the nucleoside carrying the methyl ketone function and deprotected in 5' (1.7 mmol, 85% over 2 steps) is obtained. It is carried by proton NMR, carbon 13 NMR and mass spectrometry.

Phosphorylation and production of the adenosine-(N6)-C10-methyl ketone nucleotide 2

The adenosine-(N6)-C10-methyl ketone protected in 2', 3' by isopropylidene is dissolved in anhydrous pyridine and is evaporated twice. 500 µl of pyridine, 1.5 ml of dioxane and a freshly prepared solution of 2-chloro-4H-1,2,3-dioxaphosphain-4-one (1 M) in dioxane (650 µl, 0.65 mmol) are then added under argon. Stirring is allowed to proceed for 20 minutes and then a 0.5 M solution of tributylammonium pyrophosphate in anhydrous DMF (1.6 ml, 0.8 mmol) is added simultaneously with 650 µl of tributylamine.

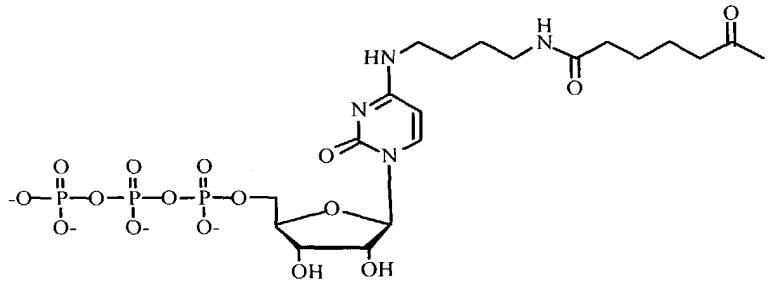
After 30 minutes, 10 ml of 1% iodine solution is added to a pyridine/water (98/2, v/v) mixture. After 20 minutes, the excess iodine is destroyed with a 5% aqueous $NaHSO_3$ solution and stirring is maintained for 10 minutes, the mixture is evaporated to dryness and water/dichloromethane extraction is performed. The aqueous phase is evaporated off and then reverse phase (C18) purification is carried out (eluent: H_2O/MeOH). The fractions containing the product are evaporated and the counter-ion exchange is carried out by passing over Dowex, Na+ resin. The protected triphosphate (0.28 mmol, 56%) is thus recovered.

<u>*Deprotection*</u>

0.035 mmol of protected triphosphate are taken up in 17.5 ml of milli-Q water, to which 17.5 ml of a 25% aqueous TFA solution are added. The mixture is stirred for 15 minutes and then evaporated and coevaporated twice with water, and the product is taken up in 10 ml of milli-Q water and neutralized with 0.1 M sodium hydroxide, to pH 8. After evaporation, a C18 purification is carried out (eluent: H_2O; H_2O/MeOH). The fractions containing the

product are evaporated and assayed. 0.022 mmol (63%) of the adenosine-(N6)-C10-methyl ketone triphosphate 2 are thus obtained.

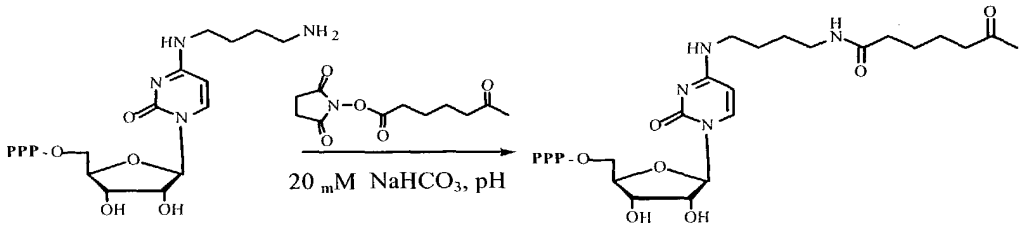
I.3. Synthesis of cytidine-(N4)-C10-methyl ketone 3



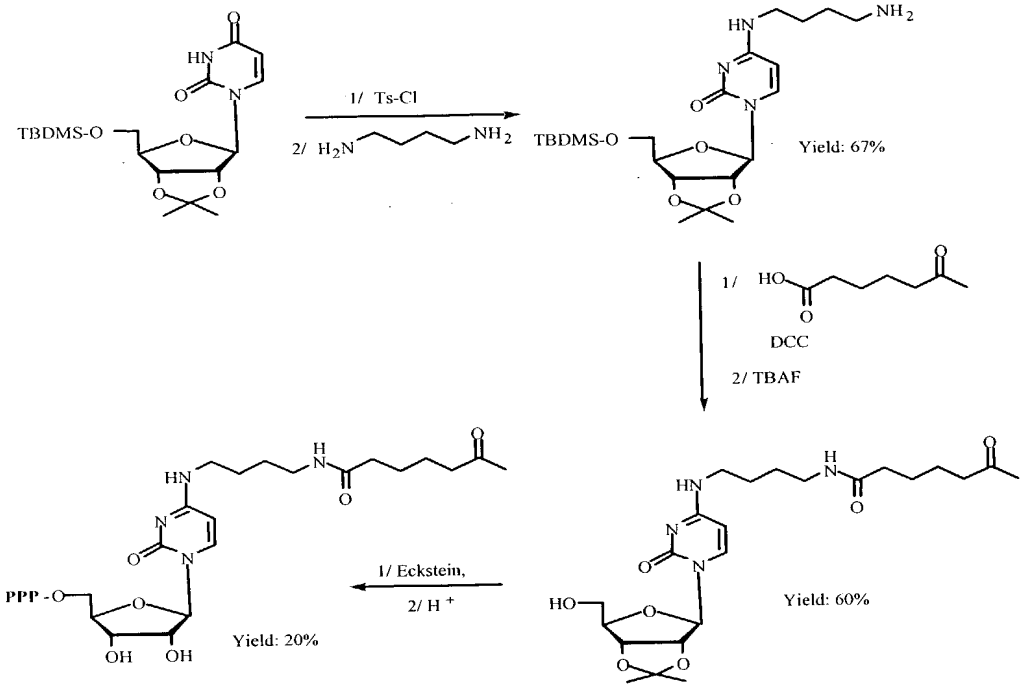
3

This nucleotide was prepared in two ways:

- *Nucleotide pathway*: coupling between an activated ester of the methyl ketone chain and aminated cytidine triphosphate.



- *Nucleoside pathway*: synthesis of the methyl ketone nucleoside then phosphorylation by the Eckstein method.

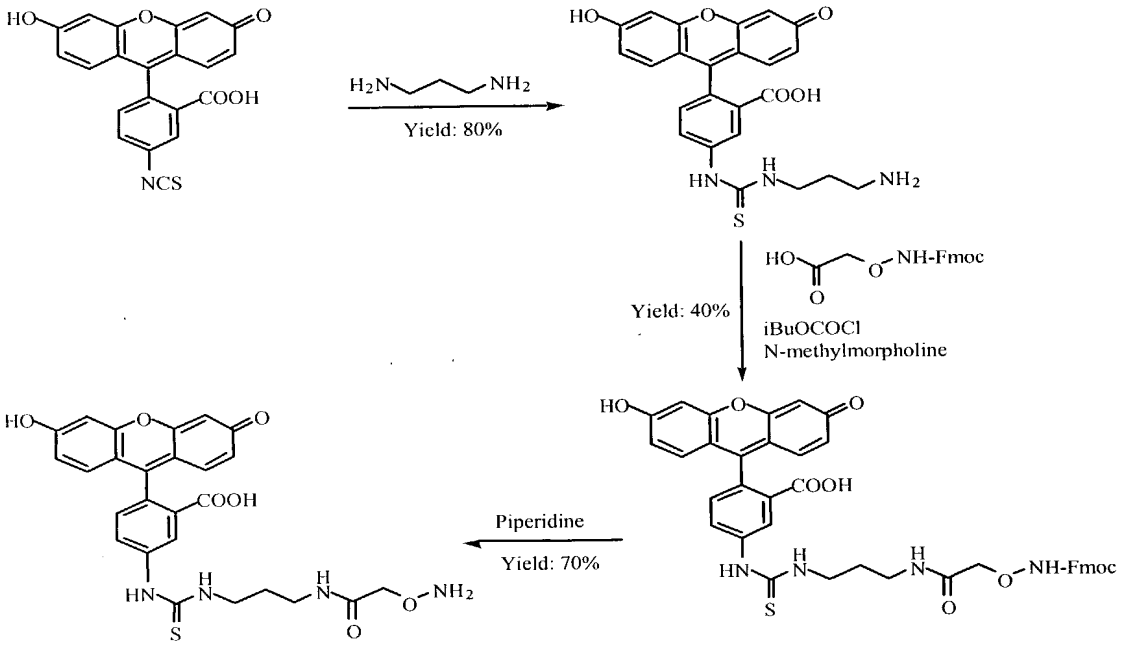


EXAMPLE II : SYNTHESIS OF THE OXYAMINE FLUOROPHORE

4

II. 1. Synthetic schemes

The oxyamine chain is introduced onto the fluorescein in three steps: the first step is nucleophilic addition of 1,3-diaminopropane to fluorescein isothiocyanate (FITC). After reverse phase purification, the oxyamine unit protected in the form of Fmoc is introduced by peptide coupling. The free oxyamine is generated by deprotection in basic medium.



4

Introduction of the 1,3-diaminopropane chain:

Diaminopropane (585 µl, 6.96 mmol) is added to 20 ml of anhydrous DMF. Fluorescein isothiocyanate (FITC) (500 mg, 1.16 mmol), solubilized in 7 ml of anhydrous DMF, is then added, dropwise, under argon. Stirring is maintained for 15 minutes after the FITC has been added. The mixture is evaporated to dryness and coevaporated twice with water. The residue is reverse phase (C18) chromatographed: (eluent: H_2O/MeOH: 1/1).

The product is thus recovered in the form of an orange powder (420 mg, 0.93 mmol, 80%). It is characterized by proton NMR, carbon 13 NMR and mass spectrometry.

Introduction of the protected alkoxyamine group:

Carboxyalkoxyamine protected with Fmoc: HOOC-CH_2-ONH-Fmoc (473 mg, 1.5 mmol in 10 ml of anhydrous DMF) is

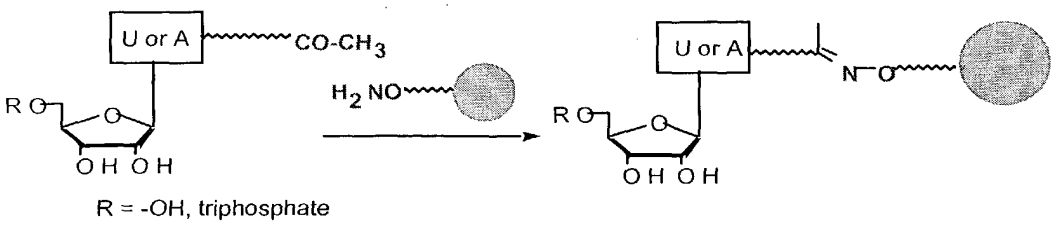
solubilized. It is placed at 0°C under argon. N-Methylmorpholine (166 µl, 1.5 mmol) is then added and, after 15 minutes, the fluorescein carrying the diaminopropane chain (350 mg, 0.75 mmol) is added. After reacting for 1 hour, the DMF is evaporated off to dryness. The residue obtained is chromatographed on silica gel (CH_2Cl_2/MeOH: 85/15 (solid deposit)). The protected fluorescein-alkoxyamine is thus obtained in the form of an orange powder (227 mg, 0.3 mmol, 40%). It is characterized by proton NMR, carbon 13 NMR and mass spectrometry.

*Production of the fluorescein-alkoxyamine label **4**:*

The fluorescein protected with fmoc (100 mg, 0.13 mmol) is solubilized in 2 ml of anhydrous DMF. Pyridine (20 µl, 0.2 mmol) is then added. After 15 minutes, the mixture is evaporated to dryness and then C18 reverse phase purification is carried out (eluent: H_2O/CH_3CN: 1/1 then CH_3CN). After evaporation, the product is obtained in the form of an orange powder (49 mg, 0.09 mmol, 70%). This fluorophore was characterized by proton NMR, 13C NMR and mass spectrometry.

EXAMPLE III : REACTIVITY OF THE METHYL KETONE COMPOUNDS WITH THE FLUOROPHORE

The reactivity was tested at the nucleoside level and at the nucleotide level:



R = -OH, triphosphate

The reaction is carried out in the presence of 1.1 eq. of fluorophore-ONH_2 (**4**) with respect to the methyl ketone compound. The reaction is rapid and selective. At the nucleoside level, the adducts were characterized by proton NMR and mass spectrometry.

EXAMPLE IV : INCORPORATION OF THE URIDINE-METHYL KETONES AND POST-TRANSCRIPTION LABELING

IV.1. Description of the main steps

Transcriptions

The transcriptions were carried out on a PCR target (fragment of the 16 S RNA of *Mycobacterium tuberculosis* (Mtb)) (Troesch A. et al, J. Clin. Microbiol., 37(1), 49-55, 1999) or a fragment of HIV reverse transcriptase (Kozal M.J. et al, Nature Medecine, 2(7), 753-759, 1996) using T7 RNA polymerase and various ratios between the functionalized nucleotide and the natural nucleotides, while at the same time keeping the total concentration of each nucleotide at 1 mM. This ratio between the functionalized nucleotide and the corresponding natural nucleotide is expressed as a percentage and the ratios used are in general 0, 30, 70 and 100%. The point 0% is used as a transcription control since, in this case, there is no functionalized nucleotide and the transcription reaction comprises the 4 natural nucleotides. When the ratio is 100%, this means that the functionalized nucleotide represents 100% of the

nucleotide studied (the other three nucleotides required for the transcription reaction naturally being the natural nucleotides). This ratio of 100% for a functionalized nucleotide is the most significant test of the neutrality with respect to an enzymatic reaction since the enzyme must incorporate this nucleotide in order to function correctly. The incubation time for the transcription reaction is 1 h at 42°C.

The transcriptions are analyzed by polyacrylamide gel electrophoresis under denaturing conditions (6% acrylamide, 7 M urea, 1XTBE). The volume loaded is 5 μl and the migration takes place for 45 min at 150 V. The transcripts, natural or functionalized with the methyl ketone function, are visualized under a UV lamp after staining with ethidium bromide.

Assay

The amount of transcripts produced in each reaction is determined by UV assay, after purification, of an aliquot derived from a transcription reaction.

Enzymatic digestion

The transcripts are purified on microcon-50 filters (Amicon, Beverly, MA) in order to remove the excess of unincorporated nucleotides. They are then hydrolyzed according to the protocol described in patent application WO 98/05766 using P1 nuclease (Boehringer reference 2362251,2U, 2 h at 37°C) and alkaline phosphatase (Boehringer-Mannheim reference 713023,1U, 1 h at 37°C). The digestions are carried out on 4×10^{14} transcript copies. The nucleoside composition is determined by reverse phase HPLC, by comparing with nucleoside standards composed of a mixture of natural nucleosides.

The HPLC conditions are as follows:

- analytical C18 column (250 × 4.6 mm) heated to 45°C,

- eluents: A: 50 mM sodium phosphate buffer, pH 7; B: MeOH/H_2O: 95/5, v/v

- gradient: 0% of B for 10 min, up to 30% of B in 10 min, up to 80% of B in 10 min, 5 min at 80% of B, up to 100% of B in 2 min.

Labeling

The transcripts are labeled using various proportions of fluorophore. The reaction time is 30 min at room temperature. The labeling is carried out on the transcripts generated from an Mtb target and/or HIV target, obtained by incorporation of 100% of a methyl ketone nucleotide. Initially, the labeled transcripts are analyzed by polyacrylamide gel electrophoresis and visualized under UV, before and after staining with ethidium bromide.

Cleavage

Before hybridizing on the DNA chip, the labeled transcripts are cleaved at 65°C for 30 min using imidazole and manganese chloride ($MnCl_2$) each at a concentration of 30 mM.

Hybridization on a DNA chip

After cleavage, the fragments obtained are hybridized, detected and analyzed on a DNA chip (Affymetrix, Santa Clara, CA, USA) according to the protocol supplied by the manufacturer.

The "myco" chips are designed for resequencing the 213-415 region of the "Genbank" M20940 sequence of the 16S

RNA of *Mycobacterium tuberculosis* (Troesch A. et al, J. Clin. Microbiol., 37(1), 49-55, 1999). Those termed "HIV PRT 440" are designed for resequencing the RT (reverse transcriptase) and protease regions of the HIV virus (Kozal M.J. et al, Nature Medecine, 2(7), 753-759, 1996).

In the case of the Mtb transcript, 10^{14} copies are hybridized on the chip.

In the case of the HIV transcript, 5×10^{12} copies are hybridized on the chip.

IV. 2. Results

Incorporation of the uridine(C5)-C9-methyl ketone nucleotide 1 (U-$COCH_3$)

Target used	Ratio between functionalized nucleotide U-$COCH_3$ and natural nucleotide	Transcripts produced (copies/microliters)
Mtb	0%	5.0^{E}+13
	30%	5.0^{E}+13
	70%	3.0^{E}+14
	100%	0.8^{E}+14
HIV	0%	0.9^{E}+12
	70%	0.9^{E}+12
	100%	$0,9^{E}$+12

The results in the table above show that the uridine-methyl ketone nucleotide (1) is very well incorporated by T7 RNA polymerase. This allows the production of activated transcripts capable of reacting with a label carrying the alkoxyamine function.

Evaluation of the incorporation by HPLC

The peak which corresponds to the U-methyl ketone nucleoside (retention time 26.2 minutes) is indeed observed in the chromatographic profile of the hydrolyzate of the transcripts containing 100% of U-$COCH_3$ (1) and obtained from the Mtb target, which means that the nucleotide is not modified during the transcription and enzymatic digestion steps. The absence of the peak corresponding to the natural uridine (retention time 14.97 minutes) is also noted.

This shows that the U-methyl ketone nucleotide is correctly incorporated and can be used at 100% in a transcription reaction.

Evaluation of the labeling by polyacrylamide gel

The labeling was carried out on the crude transcripts generated from an Mtb target, using 100% of the U-$COCH_3$ nucleotide (1). The amount of fluorophore compared to the number of reactive sites was varied. These ratios are: 2, 5, 10, 15, 20 and 50 equivalents of flurophore-ONH_2. 10 equivalents are suitable for intensive and selective labeling.

On a gel, before staining with ETB, representing labeling using 5, 10 and 15 eq. of the fluorophore, labeling is observed which is more or less intense depending on the number of equivalents of fluorophore used, but there is a visible band whatever the amount of labels used. The appearance of new bands is also noted at high doses of fluorophore. This is certainly due to a high density of the label on the target.

Hybridization and analysis of the results

The Mtb and HIV transcripts generated from corresponding targets, using 100% of UTP-$COCH_3$ (1), underwent the treatments described above. During the

cleavage step, a "blocking" agent (acetone or glutaraldehyde) was used in order to avoid saturating the chip with the excess of fluorophore-ONH_2 which adsorbs to the surface of this chip. The hybridization is carried out using the "Gene Chip fluidics Station" for hybridization (800101 Affymetrix, Santa Clara, CA), the suitable chips and buffers and the protocol supplied by the manufacturer.

The base call percentage, mean signal intensity, median intensity and background noise parameters were calculated using the software provided by the manufacturer (GenChip sequence analysis system, reference 900135, Affymetrix, Santa Clara, CA). The results are given in the two tables below. The signal intensity is expressed in RFU (fluorescence units of the manufacturer).

Mtb transcripts:

Target	Base call (%)	Mean intensity (Rfu)	Median intensity (Rfu)
• ***Mycobacterium tuberculosis*** *100% U-$COCH_3$ transcripts*			
100 eq Fluo-ONH_2 + 100 eq acetone	97.7	3540	3260
20 eq Fluo-ONH_2 + 20 eq glutaraldehyde	92.4	12630	11970

Base call: percentage of bases correctly identified.

HIV transcripts

Target	Base call (%)	Mean intensity (Rfu)	Median intensity (Rfu)
• ***HIV***			
70% U-$COCH_3$ transcripts 10 eq Fluo-ONH_2 + 10 eq glutaraldehyde	98.8	2175	1765
100% U-$COCH_3$ transcripts 10 eq Fluo-ONH_2 + 10 eq glutaraldehyde	99.0	2675	2090
100% U-$COCH_3$ transcripts 5 eq Fluo-ONH_2 without blocking	97.5	4750	4095
100% U-$COCH_3$ transcripts 5 eq Fluo-ONH_2 + 5 eq glutaraldehyde	98.5	1825	1550
100% U-$COCH_3$ transcripts 2 eq Fluo-ONH_2 without blocking	98.3	2380	2035
100% U-$COCH_3$ transcripts 2 eq Fluo-ONH_2 + 2 eq glutaraldehyde	98.8	620	540

In both the Mtb and HIV cases, very effective labeling with intensities greater than 1000 Rfu and resequencing (base call) percentages close to 100% are observed. These results show that the reaction between the alkoxyamine and methyl ketone, demonstrated at the monomer stage, is therefore specific and effective between RNA sequences containing methyl ketone functions and the fluorescent label carrying the alkoxyamine function. Specifically, a sufficient amount of labeled and detectable transcript fragments are obtained with only 2 equivalents of alkoxyamine label per methyl ketone function.

EXAMPLE V : INCORPORATION OF THE CYTIDINE-METHYL KETONE 3 AND POST-TRANSCRIPTION LABELING

The cytidine 3 was incorporated at 100% into RNAs transcribed from Mtb, as described in Example III. The cleavage and labeling method is also carried out as indicated above.

Target	Base call (%)	Mean intensity (Rfu)	Median Intensity (Rfu)
100% C-$COCH_3$ transcripts 20 eq Fluo-ONH_2 + 20 eq glutaraldehyde	94.2	6655	6200

Here again, very effective labeling is observed. The signal intensity is greater than 6000 Rfu and the resequencing is 94%. As in the previous example, this result demonstrates that the reaction between the alkoxy-amine and methyl ketone is specific and effective between RNA sequences containing methyl ketone functions and the fluorescent label carrying the alkoxyamine function.

EXAMPLE VI : ADVANTAGES OF THE METHYL KETONE FUNCTION COMPARED TO ALKOXYAMINE, ALDEHYDE AND AMINE FUNCTIONS.

1. Comparison between the alkoxyamine and methyl ketone function on the U nucleotide.

Uridine carrying an alkoxyamine chain at position 5 is prepared according to the method described in Example 4 of patent application WO-A-98/05766. The synthesis of the fluorescein aldehyde (FLUO-CHO) label is described in Example 18 of application WO-A-98/05766.

It is important to note that the alkoxyamine function requires permanent protection with the tert-butoxycarbonyl (BOC) group. This function is deprotected at the same time as the isopropylidene in 2',3' after phosphorylation in the presence of 50% trifluoroacetic acid. This percentage of TFA is necessary to obtain a deprotection of the alkoxyamine close to 90%. In order to obtain total deprotection, more concentrated acid solutions are necessary. In these solutions, the triphosphate degrades very rapidly to diphosphate and monophosphate.

In the case of the methyl ketone function, no deprotection is necessary and only 25% of TFA is used for total deprotection of the isopropylidene in 2',3' after phosphorylation. No degradation is observed with this percentage of TFA.

The methyl ketone chain is sufficiently hydrophobic even after deprotection; it thus contributes to good separation of the nucleotide triphosphate when it is purified by reverse phase HPLC. This separation is more difficult in the case of the alkoxyamine chain.

Incorporation and post-labeling of the UTP-alkoxyamine

The reactions for transcription, enzymatic hydrolysis of the transcripts, HPLC analysis and post-labeling are carried out under the same conditions as those described in Example IV.

The figure shows the transcription yields measured in terms of the amount of amplicons produced (on the

y-axis in copies/microliters) as a function of the 3 nucleotides used.

In this example, the incorporation of the two nucleotides, alkoxyamine ($U\text{-}ONH_2$) and methyl ketone ($U\text{-}COCH_3$ compound 1), is studied.

The uridine-methyl ketone 1 at 100% has no effect on the transcription yield. This yield is lower when the same nucleotide carrying the alkoxyamine function is incorporated.

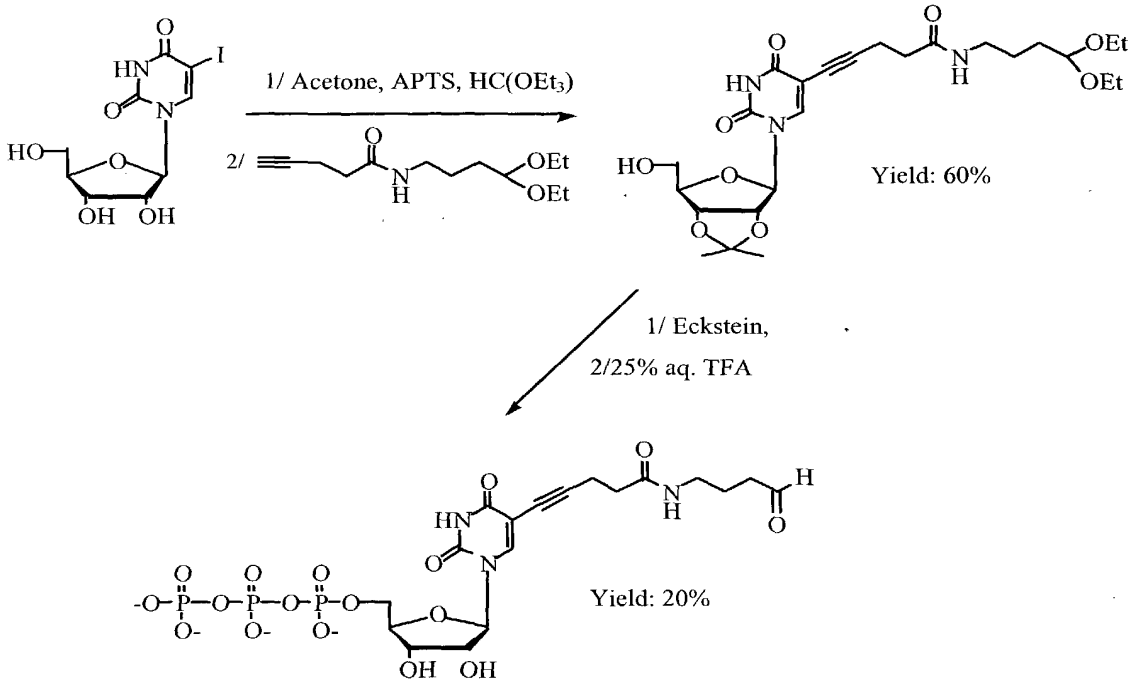
Post-labeling:

MTB TARGET	Base call (%)	Mean intensity (Rfu)	Median intensity (Rfu)
100% U-ONH2 TRANSCRIPTS + 10 EQ FLUO-CHO	91.6	5735	5335

The resequencing percentage using the uridine-alkoxyamine is lower than that obtained with its homolog carrying the methyl ketone function.

2. Comparison between the aldehyde and methyl ketone function on the U nucleotide.

The uridine with an aldehyde chain (U-CHO) is prepared according to the following scheme:



The chain is synthesized by peptide coupling between 4-aminobutyraldehyde diethylacetal and 4-pentynoic acid. Heck coupling produces the protected nucleoside. Phosphorylation followed by deprotection in acid medium correctly produces the expected nucleotide. The products were characterized by ^{1}H NMR and ^{13}C NMR.

Here again, it is necessary for the aldehyde function to be protected during the coupling and phosphorylation steps. The deprotection of the aldehyde is carried out at the same time as that of the isopropylidene (25% of TFA); however, the purification and desalination of the deprotected product are more difficult compared to those of the nucleotide-methyl ketone (U-$COCH_3$, compound 1). The yield from the phosphorylation step is 20%.

The HPLC analysis of the transcripts containing the UTP-aldehyde, hydrolyzed using the enzymatic technique

described in Example IV, shows that the nucleotide-aldehyde is transformed. This transformation is certainly due to the aldehyde function interacting with components of the buffers during the transcription or enzymatic hydrolysis of the transcripts.

The post-labeling reaction using the fluorescein-alkoxyamine shows no result after analysis of the mycobacterium transcripts on gel and on DNA chips. This shows that transformation of the aldehyde has occurred during the transcription step, which shows the advantage of the methyl ketone function compared to the aldehyde.

3. Comparison between the amine and methyl ketone function on the U nucleotide.

The uridine carrying an amine chain at position 5 (U-NH_2) is prepared according to the method described in Example 3 of patent application WO 98/05766. It is necessary to protect the amine function with the Boc group in this case as well.

The transcription yields obtained with 100% of U-NH_2 are much lower than when the methyl ketone function is used and do not allow efficient resequencing on the DNA chip both in the case of Mtb and in the case of HIV.

4. Comparison between the amine and methyl ketone function on the C nucleotide.

The cytidine carrying an amine chain at position 4 (C-NH_2) is prepared according to Example 16 of patent application WO-A-98/05766.

This nucleotide was incorporated into fragments of the 16S RNA of *Mycobacterium tuberculosis* through

transcription reactions carried out on a PCR target according to the protocol described in Example IV.

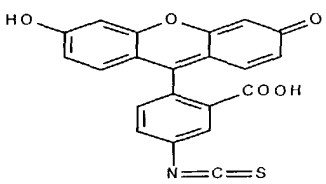
The HPLC analysis of the transcripts containing the C-NH_2, hydrolyzed using the enzymatic technique described in the example above, shows that the aminated nucleotide is correctly incorporated and is intact.

The yields from transcription with various percentages of C-NH_2, also determined as indicated in Example IV, are given in the table below.

Ratio between functionalized *nucleotide* C-NH_2 and natural nucleotide (%)	Transcripts produced (copies / microliters)
0	$3.48^{E}+13$
70	$1.94^{E}+13$
100	$1.51^{E}+12$

It is important to note that, with 100% of C-NH_2, the amount of amplicons obtained is 20 times less than that obtained with transcription containing 100% of natural nucleotide.

The labeling reagent used in the post-labeling reaction was fluorescein isothiocyanate obtained from Sigma-Aldrich (St Quentin Falavier, France).



Fluorescein isothiocyanate (FITC)

The labeling of the transcripts obtained by incorporation of the C-NH_2 were labeled with FITC, hybridized on the DNA chip intended for the identification of these Mtb targets, and the signals were analyzed according to the protocol described above. The results obtained with and without blocking agent are given in the table above .

Target	Base call (%)	Mean intensity (Rfu)
Nonpurified transcripts + 100 eq FITC	ND	ND
Nonpurified transcripts + 100 eq FITC +1000 eq of blocking diamine	86	346

ND : not determined, the surface of the chip is completely saturated.

Blocking diamine : H_2N-CH_2-CH_2-O-CH_2-CH_2-O-CH_2-CH_2-NH_2.

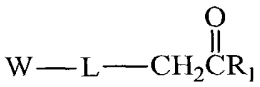
If the diamine chain is not added after the labeling, the surface of the chip is completely saturated with the 100 eq of label and the signals cannot be exploited. This saturation is certainly due to the interaction of the isothiocyanate function with the exocyclic amine functions of the bases.

The post-labeling addition of 1000 eq of diamine, having the same structure as that carried by the cytidine, appears to confirm this hypothesis since the surface of the chip is no longer saturated, and 86% resequencing is obtained. The signal intensity is low compared to that obtained with transcripts containing the methyl ketone and labeled with fluorescein-alkoxyamine.

In addition to the inhibitory effect of the $C-NH_2$ during the transcription, the resequencing percentage when the $C-NH_2$ is used is lower than that obtained with its homolog carrying the methyl ketone function ($C-COCH_3$, compound 3).

CLAIMS

1/ Functionalized compound of general formula (I) :



(I)

in which

W represents a nucleotide analog,

L represents a linker arm comprising at least four atoms,

R_1 represents a linear or branched alkyl chain.

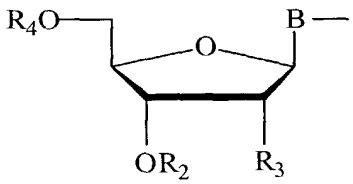
2/ Compound according to claim 1, characterized in that R_1 represents an alkyl chain having at most 6 carbon atoms.

3/ Compound according to claim 2, characterized in that R_1 represents a methyl group.

4/ Compound according to any one of claims 1 to 3, characterized in that L comprises at least eight atoms.

5/ Compound according to any one of claims 1 to 4, characterized in that L is a saturated or unsaturated hydrocarbon-based chain, optionally interrupted by at least one function chosen from amine, amide and oxy functions.

6/ Compound according to any one of claims 1 to 5, characterized in that W corresponds to the general formula (II)



(II)

in which:

- R_2 represents H or a protective group,

- R_3 represents H, F, OH, SH, NH_2, OCH_3 or OR_5 in which R_5 represents a protective group or an alkyl chain,

- R_4 represents an H radical, a protective group or a mono-, di- or triphosphate group,

- W being attached to L via B.

7/ Compound according to claim 6, characterized in that the nitrogen-containing base is cytosine, uracil or adenine.

8/ Compound according to claims 6 and 7, characterized in that R_2 is an H, R_3 is an OH group and R_4 is a triphosphate group.

9/ Compound according to claims 6 and 7, characterized in that R_2 is a 2-cyanoethyl-N,N-diisopropylphosphoramidite group and R_3 is H or OR_5 in which R_5 is a protective group used in oligoribonucleotide synthesis and R_4 is a 4,4'-dimethoxytrityl group.

10/ Functionalized polynucleotide comprising at least one functionalized compound according to any one of the preceding claims.

11/ Functionalized polynucleotide according to claim 10, characterized in that this polynucleotide is prepared by the chemical and/or enzymatic route.

12/ Functionalized polynucleotide according to claim 11, characterized in that this polynucleotide is prepared using an enzymatic amplification reaction.

13/ Labeled functionalized polynucleotide, characterized in that it comprises at least one functionalized compound of general formula (I'):

$$W-[-L-]_n-CH_2COR_1 \qquad (I')$$

in which

W represents a nucleotide analog,

L represents a linker arm comprising at least four atoms,

n represents an index equal to 0 or 1,

R_1 represents a linear or branched alkyl chain,

the alkyl ketone group of said functionalized compound having interacted with a labeling reagent.

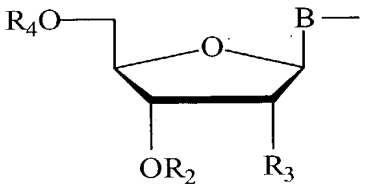
14/ Polynucleotide according to claim 13, characterized in that R_1 represents an alkyl chain having at most 6 carbon atoms.

15/ Polynucleotide according to claim 14, characterized in that R_1 represents a methyl group.

16/ Polynucleotide according to any one of claims 13 to 15, characterized in that L comprises at least eight atoms.

17/ Polynucleotide according to any one of claims 13 to 16, characterized in that L is a saturated or unsaturated hydrocarbon-based chain, optionally interrupted by at least one function chosen from amine, amide and oxy functions.

18/ Polynucleotide according to any one of claims 13 to 17, characterized in that W corresponds to the general formula (II)



(II)

in which:

- R_2 represents H or a protective group,
- R_3 represents H, F, OH, SH, NH_2, OCH_3 or OR_5 in which R_5 represents a protective group or an alkyl chain,
- R_4 represents an H radical, a protective group or a mono-, di- or triphosphate group,
- W being attached to L via B.

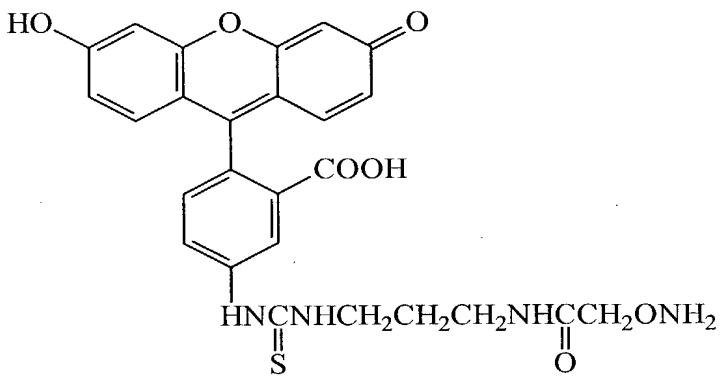
19/ Polynucleotide according to claim 18, characterized in that the nitrogen-containing base is cytosine, uracil or adenine.

20/ Polynucleotide according to claims 18 and 19, characterized in that R_2 is an H, R_3 is an OH group and R_4 is a triphosphate group.

21/ Compound according to claim 18 and 19, characterized in that R_2 is a 2-cyanoethyl-N,N-diisopropylphosphoramidite group and R_3 is H or OR_5 in which R_5 is a protective group used in oligoribonucleotide synthesis and R_4 is a 4,4'-dimethoxytrityl group.

22/ Polynucleotide according to any one of claims 13 to 21, characterized in that the labeling reagent comprises a hydrazine or alkoxyamine function.

23/ Polynucleotide according to claim 22, characterized in that the labeling reagent is



24/ Method for detecting a target nucleic acid, characterized in that this target nucleic acid is brought into contact with at least one functionalized nucleotide as defined in any one of claims 13 to 21, in the presence of elements and under conditions required for producing a polynucleotide, so as to produce a functionalized polynucleotide; the polynucleotide obtained is labeled with a labeling reagent; and then said labeled polynucleotide is detected.

25/ Method according to claim 24, characterized in that the functionalized polynucleotide is obtained using an enzymatic amplification reaction.

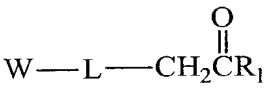
26/ Method for detecting a target nucleic acid, characterized in that this target nucleic acid is brought into contact with a functionalized polynucleotide according to any one of claims 10 to 12; the labeling reagent is reacted; and the presence of the target nucleic acid is detected.

27/ Method for detecting a target nucleic acid, characterized in that a labeled polynucleotide according to any one of claims 13 to 23 is available for use, this target nucleic acid is brought into contact with the

labeled polynucleotide; and the presence of the target nucleic acid is detected.

A B S T R A C T

The invention relates to a functionalized compound of general formula (I):



(I)

in which

W represents a nucleotide analog,

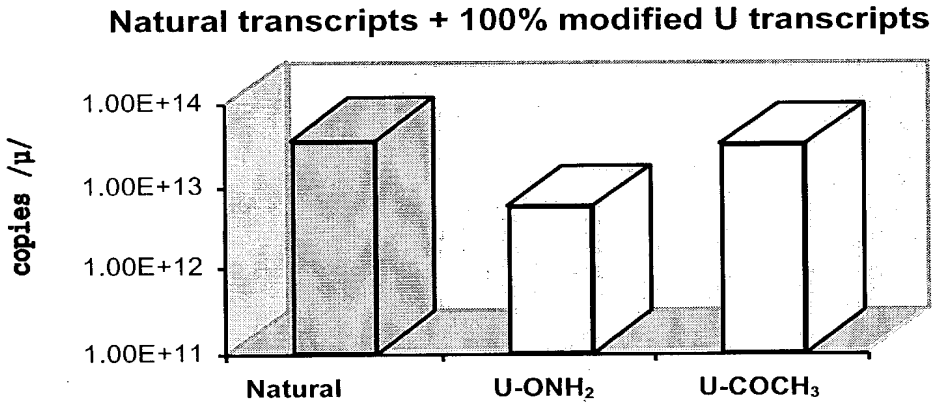
L represents a linker arm comprising at least four atoms,

R_1 represents a linear or branched alkyl chain,

A functionalized polynucleotide comprising at least one said compound and a labeled functionalized polynucleotide comprising at least one functionalized compound corresponding to the formula (I').

A method for detecting a target nucleic acid using a said compound is described.

FIGURE


Natural transcripts + 100% modified U transcripts
copies /µl/
1.00E+14
1.00E+13
1.00E+12
1.00E+11
Natural
U-ONH2
U-COCH3

09869796 .091901

Docket No.: 110024

DECLARATION AND POWER OF ATTORNEY UNDER 35 USC §371(c)(4) FOR PCT APPLICATION FOR UNITED STATES PATENT

As a below named inventor, I hereby declare that:
my residence, post office address and citizenship are as stated below under my name;

I verily believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought, namely the invention entitled: FUNCTIONALISED POLYNUCLEOTIDE COMPOUND, OPTIONALLY MARKED AND METHOD FOR DETECTING A TARGET NUCLEIC ACID
described and claimed in international application number PCT/FR00/00011 filed January 5, 2000 .

I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations §1.56.

Under Title 35, U.S. Code §119, the priority benefits of the following foreign application(s) filed by me or my legal representatives or assigns within one year prior to my international application are hereby claimed:

French Patent Application No. 99.00111 filed January 5, 1999.

The following application(s) for patent or inventor's certificate on this invention were filed in countries foreign to the United States of America either (a) more than one year prior to my international application, or (b) before the filing date of the above-named foreign priority application(s):

I hereby appoint the following as my attorneys of record with full power of substitution and revocation to prosecute this application and to transact all business in the Patent Office:

James A. Oliff, Reg. No. 27,075; William P. Berridge, Reg. No. 30,024;
Kirk M. Hudson, Reg. No. 27,562; Thomas J. Pardini, Reg. No. 30,411;
Edward P. Walker, Reg. No. 31,450; Robert A. Miller, Reg. No. 32,771;
Mario A. Costantino, Reg. No. 33,565; Stephen J. Roe, Reg. No. 34,463;
Joel S. Armstrong, Reg. No. 36,430; Christopher W. Brown, Reg. No. 38,025; and
Richard E. Rice, Reg. No. 31,560.

ALL CORRESPONDENCE IN CONNECTION WITH THIS APPLICATION SHOULD BE SENT TO OLIFF & BERRIDGE, PLC, P.O. BOX 19928, ALEXANDRIA, VIRGINIA 22320, TELEPHONE (703) 836-6400.

I hereby declare that I have reviewed and understand the contents of this Declaration, and that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1 ***Typewritten Full Name of Sole or First Inventor*** 100 Eric (Given Name) — Middle Initial — DEFRANCQ (Family Name)

2 **Inventor's Signature** Eric Defrancq

3 **Date of Signature** July (Month) 25 (Day) 2001 (Year)

Residence: Concelin (City) — (State or Province) — FRANCE FRX (Country)

Citizenship: FRANCE

Post Office Address: (Insert complete mailing address, including country) Grande Rue
38570 Concelin FRANCE

Note to Inventor: Please sign name on line 2 exactly as it appears in line 1 and insert the actual date of signing on line 3.

IF THERE IS MORE THAN ONE INVENTOR USE PAGE 2 AND PLACE AN "X" HERE ☒

(Discard this page in a sole inventor application)

1 ***Typewritten Full Name of Joint Inventor*** 200 Ali (Given Name) | (Middle Initial) | LAAYOUN (Family Name)

2 **Inventor's Signature:** Ali Laayoun

3 **Date of Signature:** July (Month) 23 (Day) 2001 (Year)

Residence: Lyon (City) | (State or Province) | FRANCE (Country) FRX

Citizenship: FRANCE

Post Office Address: (Insert complete mailing address, including country) 1 rue de Rhone, 69007 Lyon FRANCE

1 ***Typewritten Full Name of Joint Inventor*** 300 Jean (Given Name) | (Middle Initial) | LHOMME (Family Name)

2 **Inventor's Signature:** Jean Lhomme

3 **Date of Signature:** August (Month) 20 (Day) 2001 (Year)

Residence: Meylan (City) | (State or Province) | FRANCE (Country) FRX

Citizenship: FRANCE

Post Office Address: (Insert complete mailing address, including country) 13 rue des Brandons, 38240 Meylan FRANCE

1 ***Typewritten Full Name of Joint Inventor*** 400 Emmanuelle (Given Name) | (Middle Initial) | TREVISIOL (Family Name)

2 **Inventor's Signature:** Emmanuelle TREVISIOL

3 **Date of Signature:** August (Month) 29 (Day) 2001 (Year)

Residence: Grenoble (City) | (State or Province) | FRANCE (Country) FRX

Citizenship: FRANCE

Post Office Address: (Insert complete mailing address, including country) 15 Ed du Marechal Joffre, 38000 Grenoble FRANCE

1 ***Typewritten Full Name of Joint Inventor*** (Given Name) | (Middle Initial) | (Family Name)

2 **Inventor's Signature:**

3 **Date of Signature:** (Month) (Day) (Year)

Residence: (City) | (State or Province) | (Country)

Citizenship:

Post Office Address: (Insert complete mailing address, including country)

Note to Inventor: Please sign name on line 2 exactly as it appears in line 1 and insert the actual date of signing on line 3.

This form may be executed only when attached to the first page of the Declaration and Power of Attorney of the application to which it pertains.